Recapitulating synaptic vesicle fusion and neurotransmitter release on a biomimetic membrane

Transport mechanisms within cells and through the plasma membrane rely primarily on membrane-enveloped vesicles which ferry packets of enclosed cargo[1-4]. These vesicles release their cargo through a fusion pore by docking and fusing with their target membrane. A special case is that of neurotransmission in which synaptic vesicles deliver neurotransmitters at the synaptic cleft. The timing of this process is highly constrained such that the final series of events that lead to neurotransmitter release must take place on a sub-millisecond time-scale [5]. This finely-tuned fast release is controlled by a highly sophisticated molecular machinery. All components of this machinery have already been identified and their main functions have been established[6-10]. In brief, after being filled with neurotransmitters, synaptic vesicles are stored in a reserve pool from where they migrate towards an "active zone" of the neuronal presynaptic plasma membrane[5]. There, they reach an ill-defined state called "primed" state and form a readily releasable pool (RRP). When an action potential arrives, the plasma membrane depolarizes and voltage-gated calcium channels open, inducing calcium entry into the neuron. Calcium ions are recognized by a calcium sensor, Synaptotagmin 1, which changes conformation. This conformational change destabilizes the primed state such that vesicles in the RRP fuse very quickly with the plasma membrane (in less than 1 msec)[3, 11, 12] and thereby release their neurotransmitters[4, 5, 13]. Fusion is achieved upon the mechanical action of SNARE proteins (acronym of soluble N-ethylmaleimide-sensitive factor attachment protein receptors). Prior to fusion, v-SNAREs from the synaptic vesicle assemble like a zipper with t-SNAREs from the target plasma membrane to form a coiled-coil structure that brings the membranes close together and prime them for fusion[14]. The v-SNARE/t-SNARE complex, called SNAREpin, is only partly zippered in this primed state and fully assembled when fusion is completed[9, 15]. After opening of the fusion pore, neurotransmitters can freely diffuse in the synaptic cleft through the pore and reach their receptor on the plasma membrane of the next cell, another neuron or a muscle. This sequence of events is found in textbooks and widely accepted. However, the overall molecular organization, temporal orchestration and precise function of all the involved components remain unclear as attested by the numerous and sometimes conflicting articles published each year on the matter. In this PhD, we will focus on two key factors that are still poorly understood: the collective assembly of several SNAREpins and the opening of the fusion pore. These questions could not be previously addressed because of technical limitations. Several groups, including ours, have successfully monitored single SNARE-induced fusion events[16-19] in the context of supported membranes or tiny free-standing membranes. Even though each of these systems shed invaluable light on neurotransmission, they all failed to fully reconstitute the kinetics and molecular stoichiometry of the fusion process mainly because the membranes had limited fluidity or dimensions below the optical resolution, and because the acquisition rate was not sufficient. The systematic lack of follow-up in each case attests that the technical limitations of the experimental setups were reached after the first few publications. Hence, up to now, no artificial membrane platform was available to generate and perform measurements on a membrane platform fully reproducing the physiological constraints (asymmetrical composition, protein orientation, *etc.*). We have recently developed such a platform using a combination of high-end microfluidic, optical imaging and electrical measurements (admittance and current) techniques (Figure). **The goal of this PhD is to make a significant step forward in the understanding of neurotransmission by monitoring the kinetics of collective SNARE assembly and fusion pore formation.**

This platform is based on a giant (~100µm) suspended, fluid and solvent-free membrane made in a specifically designed microfluidic chip (Fig). As under physiological conditions, the membrane will be asymmetric, *i.e.* each leaflet of the lipid bilayer will be of different compositions. The buffer on either side of the membrane can also be readily changed to mimic intra- and extracellular media. Because the membrane is horizontal and floating in the vicinity of the coverslip, it can be observed by confocal microscopy. With our setup, admittance or current measurements will also be simultaneously performed. With the most recent highly sensitive and fast equipment, temporal resolutions of ~10 µsec for electrical measurements and ~10 msec for fluorescence observations (which is commensurate with the kinetics of neurotransmission) can be obtained.

Specific aims of the PhD

t-SNAREs will be inserted in the membrane with the correct orientation to obtain a t-SNARE decorated Free suspended BiLayer (t-FBL, mimicking the neuronal plasma membrane). We will add small unilamellar vesicles containing v-SNAREs (v-SUVs, mimicking synaptic vesicles) and monitor their docking and fusion to the t- FBL. To quantitatively describe the neurotransmission process, we will

focus on 2 main characteristic features of single docking/fusion events: 1. The movement of v-SUVs prior to fusion (single v-SUV) which we will correlate with the rate of multiple SNAREpin engagement; 2. The dynamics of fusion pore opening which is associated with the amount of cargo released over time.

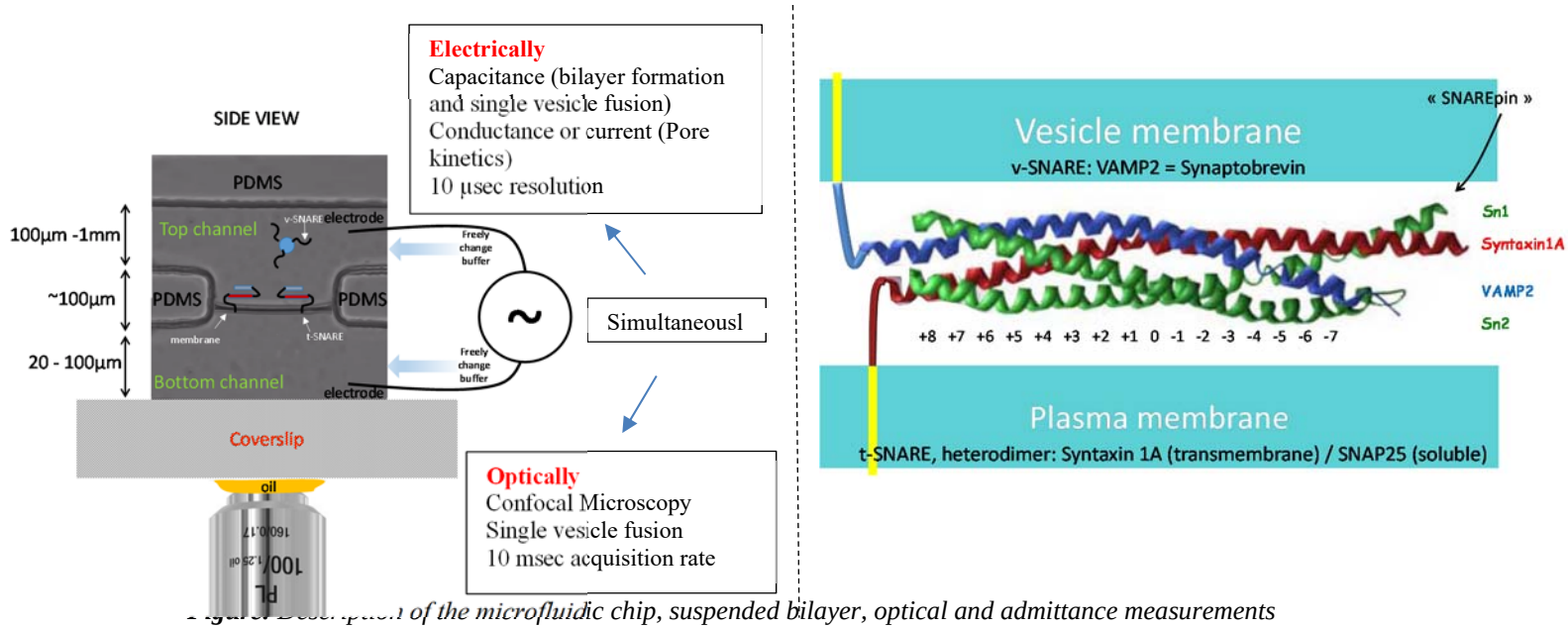

Figure 1: Description of the microfluidic chip, suspended bilayer, optical and admittance measurements

Current methods to form horizontal suspended lipid membranes have significant drawbacks such as hydrocarbon incorporation from the organic solvent, thinning time and reproducibility. We have designed a versatile platform to generate suspended, planar, hydrocarbon-free, asymmetric, and fluid membranes with any lipid/protein composition of both leaflets and possibility to indefinitely change buffer on both sides. The technique relies on a microfluidic setup in which a single block of poly(dimethylsiloxane) (PDMS) is molded to obtain two channels separated by a ~100 µm hole. The mold is made from a resin in a Stereo Lithography Apparatus (SLA) 3D printer with an accuracy of 10 µm. In the chip, oil is initially trapped in the hole between the channels and a monolayer is formed on each side. PDMS spontaneously absorbs the oil which ensures a complete removal of any hydrocarbon chain and a perfect control of the self-assembly kinetics of the two monolayers leading to a bilayer.

Left: Artificial sketch of the microfluidic setup, not to scale. The chip is placed on top of a coverslip under an inverted microscope. The hole surrounded by two portions of the PDMS film is shown in the center. The membrane, spanning through the hole, prevents any exchange between the top and bottom channel, mimicking extra- and intracellular fluids respectively. Proteins can be incorporated within the membrane (here, t-SNAREs) and solutions can be changed in both channels. In this sketch, a small vesicle (blue) containing v-SNAREs is flowed in the top channel. It will dock and fuse to the suspended membrane through the action of SNAREpins. One electrode is inserted in each channel. This unique setup allows the simultaneous monitoring of single vesicle fusion by electrical and optical measurements.

Right: SNARE complex (SNAREpin). The two SNAREs, v and t-SNAREs, zipper in a coiled-coil structure through 16 hydrophobic layers, numbered -7 to +8, and thereby force the membranes together and induce fusion.

1. Prior to fusion: Delay between docking and fusion vs. number of SNAREpins formed

We will measure the delay between the time when the v-SUV docks onto the model plasma membrane, as observed by fluorescence, and the time when fusion occurs, as observed by both fluorescence and electrical measurements. Characterizing the instantaneous diffusion coefficient of the v-SUV will indicate how many SNAREpins are formed at any given time. Indeed, the diffusion coefficient quickly decreases as several SNAREpins form. Hence, steps will be observed in the spontaneous diffusion coefficient that will be commensurate with the number of SNAREpins that are engaged.

As a result, for each monitored v-SUV, we will obtain a timeline in which the kinetics of docking, of collective SNAREpin engagement and of fusion will be precisely measured.

2. During and after fusion: Pore dynamics and cargo release

When monitoring a v-SUV, the fusion pore dynamics can be directly acquired from the conductance or the current between the two channels. The variation of the pore size over time will be correlated with the number of SNAREpins assembled at the time of fusion. It will also be compared with the ones obtained on neurons by electrophysiology[20, 21]. The kinetics of cargo release will be directly observed by fluorescence and correlated with the pore size.

Main factors that will be tested

The effect of both physiological and physical determinants on docking/fusion and pore opening will be considered. A few examples are provided below.

SNAREpin assembly

SNAREpin zipper in a coiled coil structure by sequentially binding hydrophobic layers (see figure). We will test the effect of each of these layers by systematically mutating them. Some mutations of these layers have been related to neurological pathologies, *e.g.* mutating layer "+4" leads to symptoms related to schizophrenia-related endophenotypes[22].

Plasma membrane properties

Taking advantage of the versatility of the t-FBL, we will address the long-lasting soft-matter physics questions about the role of curvature, surface tension and elastic modulus on fusion. These parameters are more and more regarded as key factors to regulate intracellular trafficking. Spontaneous curvature and elastic modulus will be changed by modifying the composition and symmetry of the membrane. We will start with symmetrical v-SUVs and t-FBL having commonly used model lipid composition (85% 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) and 15% 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS)). Then, we will move towards more physiological compositions by adding cholesterol using a cholesterol to phospholipid ratio (mol:mol) of 0.8 in v-SUV and 1 in t-FBL. In t-FBL, ~2% of POPC will be replaced by brain-L-α-phosphatidylinositol-4,5-bisphosphate (PIP2). Finally, we will test asymmetric t-FBLs with compositions mimicking that of the neuronal plasma membrane (for example, with DOPS exclusively in the inner leaflet – which faces the intracellular fluid – and/or with an enrichment in phosphatidylethanolamine in the inner vs. the outer leaflet). This step-wise complexification of the membranes will allow the determination of the role of each component.

Cytoskeleton

To test the effect of the environment, we will artificially grow an actin network on the "cytosolic" side of the t-FBL, *i.e.* the side where v-SUVs will be injected. We will also add crowding agents both in solution and in the membrane to determine the effect of the numerous macromolecules that make the cell a highly crowded environment.

Complementarity of the two laboratories where the PhD will take place

All single vesicle fusion experiments will be performed at the LPENS where the suspended membrane technology has been developed and is up-and-running. The team of F. Pincet possesses the expertise required to ensure a successful outcome.

All protein purifications and functional/biochemical characterizations will be done in the team of D. Tareste. They have a strong expertise in SNARE protein production which guarantees that biological material of high quantity and purity will be obtained by the PhD student. They also work in close collaboration with psychiatrists which may help identify relevant mutants and relate our results to neuropathologies.

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